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**Abstract:** **OBJECTIVE:** Endogenous hypercortisolism is a chronic condition associated with severe metabolic disturbances and cardiovascular sequela. The aim of this study was to characterize metabolic alterations in patients with different degrees of hypercortisolism by mass-spectrometry-based targeted plasma metabolomic profiling and correlate the metabolomic profile with clinical and hormonal data. **DESIGN:** Cross-sectional study. **METHODS:** Subjects (n = 149) were classified according to clinical and hormonal characteristics: Cushing's syndrome (n = 46), adrenocortical adenomas with autonomous cortisol secretion (n = 31) or without hypercortisolism (n = 27). Subjects with suspicion of hypercortisolism, but normal hormonal/imaging testing, served as controls (n = 42). Clinical and hormonal data were retrieved for all patients and targeted metabolomic profiling was performed. **RESULTS:** Patients with hypercortisolism showed lower levels of short-/medium-chain acylcarnitines and branched-chain and aromatic amino acids, but higher polyamines levels, in comparison to controls. These alterations were confirmed after excluding diabetic patients. Regression models showed significant correlation between cortisol after dexamethasone suppression test (DST) and 31 metabolites, independently of confounding/contributing factors. Among those, histidine and spermidine were also significantly associated with catabolic signs and symptoms of hypercortisolism. According to an discriminant analysis, the panel of metabolites was able to correctly classify subjects into the main diagnostic categories and to distinguish between subjects with/without altered post-DST cortisol and with/without diabetes in >80% of the cases. **CONCLUSIONS:** Metabolomic profiling revealed alterations of intermediate metabolism independently associated with the severity of hypercortisolism, consistent with disturbed protein synthesis/catabolism and incomplete -oxidation, providing evidence for the occurrence of metabolic inflexibility in hypercortisolism.

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# Cortisol-related metabolic alterations assessed by mass spectrometry assay in patients with Cushing's syndrome

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## Abstract

**Objective:** Endogenous hypercortisolism is a chronic condition associated with severe metabolic disturbances and cardiovascular sequela. The aim of this study was to characterize metabolic alterations in patients with different degrees of hypercortisolism by mass-spectrometry-based targeted plasma metabolomic profiling and correlate the metabolomic profile with clinical and hormonal data.

**Design:** Cross-sectional study.

**Methods:** Subjects ( $n = 149$ ) were classified according to clinical and hormonal characteristics: Cushing's syndrome ( $n = 46$ ), adrenocortical adenomas with autonomous cortisol secretion ( $n = 31$ ) or without hypercortisolism ( $n = 27$ ). Subjects with suspicion of hypercortisolism, but normal hormonal/imaging testing, served as controls ( $n = 42$ ). Clinical and hormonal data were retrieved for all patients and targeted metabolomic profiling was performed.

**Results:** Patients with hypercortisolism showed lower levels of short-/medium-chain acylcarnitines and branched-chain and aromatic amino acids, but higher polyamines levels, in comparison to controls. These alterations were confirmed after excluding diabetic patients. Regression models showed significant correlation between cortisol after dexamethasone suppression test (DST) and 31 metabolites, independently of confounding/contributing factors. Among those, histidine and spermidine were also significantly associated with catabolic signs and symptoms of hypercortisolism. According to an discriminant analysis, the panel of metabolites was able to correctly classify subjects into the main diagnostic categories and to distinguish between subjects with/without altered post-DST cortisol and with/without diabetes in >80% of the cases.

**Conclusions:** Metabolomic profiling revealed alterations of intermediate metabolism independently associated with the severity of hypercortisolism, consistent with disturbed protein synthesis/catabolism and incomplete  $\beta$ -oxidation, providing evidence for the occurrence of metabolic inflexibility in hypercortisolism.

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## Introduction

Glucocorticoids – with cortisol as the main endogenous hormone in humans – are among the most powerful metabolic regulators and most widely used anti-inflammatory drugs. They induce a plethora of physiological effects on a variety of target tissues, stimulating hepatic gluconeogenesis, inhibiting glucose uptake in adipose and muscle tissue and enhancing lipolysis (1). According to its central role in physiology, disturbances in glucocorticoid secretion or prolonged glucocorticoid therapy have unwarranted clinical consequences. In fact, endogenous hypercortisolism also referred to as Cushing's syndrome (CS) is associated with increased mortality (2), due to impaired glucose metabolism, infectious and thrombotic complications, and musculoskeletal and cardiovascular co-morbidities (3). Even in patients with milder forms of hypercortisolism, who are not clinically evident as overt CS, metabolic and cardiovascular complications are well recognized (4, 5, 6).

The diagnosis of CS is dependent on a combination of clinical and biochemical tests whose interpretation can be misleading and requires expert knowledge (7). The identification of metabolic alterations potentially related to the level of cortisol excess in patients with hypercortisolism, may aid in disease detection and classification of its severity. Moreover, the characterization of the metabolic profile of hypercortisolism may represent a useful basis to tailor targeted treatments of diseases associated with excessive exposure to glucocorticoids, in endogenous and iatrogenic CS, the latter being the most common form of hypercortisolism.

Mass spectrometry (MS)-based assays have recently revolutionized the analysis of small molecules in biological fluids, thanks to their high accuracy and sensitivity, and have been successfully employed for steroid profiling in patients with hypercortisolism (8, 9). The aim of our study was to characterize the profile of a wide panel of metabolites in plasma of patients with different degrees of endogenous glucocorticoid excess by tandem mass spectrometry (MS/MS) and to identify potential relationships with clinical phenotypes.

## Subjects and methods

### Subjects

We enrolled 104 consecutive patients evaluated in three German tertiary clinical centers. These were classified according to clinical characteristics and the results of

1-mg dexamethasone suppression test (DST) into three groups. The first group consisted of patients with CS ( $n=49$ ), diagnosed according to current guidelines (7) and included 27 with ACTH-dependent CS. Among them, three patients had ectopic CS. The remaining two groups consisted of patients with incidentally discovered benign adrenocortical tumors, who had no typical signs/symptoms of CS and either post-DST cortisol  $\leq 50$  nmol/L ( $1.8 \mu\text{g/dL}$ ) (non-secreting – NS,  $n=27$ ) or  $>50$  nmol/L (autonomous cortisol secretion – ACS,  $n=31$ ), according to the current European guidelines (10). Controls were selected among individuals consecutively evaluated for an initial clinical suspicion of hypercortisolism (mainly because of weight gain or hypertension), in whom cortisol hypersecretion was ruled out by appropriate hormonal testing ( $n=42$ ). All subjects gave written informed consent for the analysis. The ethics committee of each individual institution approved the study.

### Clinical and hormonal evaluation

Clinical and hormonal data of patients were drawn from the German Cushing's registry (CUSTODES) and the European Network for the Study of Adrenal Tumors (ENSAT) database. Plasma cortisol, ACTH and midnight salivary cortisol were measured as described previously (11). Post-DST cortisol was measured during the morning, 8–10h after oral administration of 1-mg of dexamethasone, and levels  $<50$  nmol/L were considered adequately suppressed.

### Sample preparation and metabolomic profiling

Plasma was extracted from blood samples obtained after overnight fasting, following standard operating procedures. After centrifugation at room temperature for 10 min at  $2750g$ , samples were transferred into pre-cooled collection tubes, vortexed, aliquoted on ice, immediately frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until assayed.

Targeted metabolite quantification was performed using the AbsoluteIDQ-p180 Kit (BIOCRATES AG, Austria). Details on assay methods have been previously described (12). Briefly, targeted metabolite consists of a HPLC separation step (LC) and a flow injection analysis step (FIA), followed by MS analyses (LC-MS/MS and FIA-MS/MS respectively). The LC-MS/MS procedure enables quantification of 21 amino acids and 21 biogenic amines. The FIA-MS/MS procedure quantifies 146 additional

metabolites, covering free carnitine, 39 acylcarnitines, 38 diacyl- (PC aa), 38 acyl/alkyl- (PC ae) and 14 lyso- (lyso PC) phosphatidylcholines, 15 sphingomyelins (SM) and sum of hexoses. Limits of detection were set to three times the concentration values of the zero samples (PBS). Internal standards served as reference for calculation of metabolite concentrations given in  $\mu\text{M}$ . For quality control and normalization, five samples of the same human reference plasma have been measured with each batch of measurement. Quality control of metabolomic dataset was performed as previously described (12).

### Statistical analysis

Data are presented as mean  $\pm$  S.D., if not otherwise specified, or frequencies. Data normalization was performed by dividing each metabolite concentration by the mean of the concentration of reference plasma of the respective plate, to reduce inter-assay variability. In case of non-normal distribution, logarithmic transformation was performed. Continuous variables' differences among groups were tested with one-way ANOVA. ANCOVA was performed to adjust for the effect of age in continuous variables' comparison among groups. Selected inter-group differences were analyzed by simple contrasts. Chi-square test was performed for analysis of categorical variables. The Benjamini-Hochberg procedure was applied to control the false discovery rate (FDR) at

$\alpha$  level 0.1. Discriminant analysis was performed to assess the predictive scores for classification of patients into the different diagnostic categories and into four newly defined groups, based on the presence/absence of diabetes, and post-DST cortisol levels below/above 50 nmol/L. A ROC curve was built to test the sensitivity and the specificity of a predictive score obtained by the analysis of coefficients of the most discriminant metabolites. Regression models were built to assess the relationship between each metabolite with post-DST cortisol, including age, body mass index (BMI), diabetes and dyslipidemia. The relationship between a selected panel of metabolites and catabolic signs of hypercortisolism was investigated by multinomial logistic regression, including age and diabetes into the model. Statistical analysis was performed with SPSS, version 23 (IBM). 3D scatterplots were prepared using JMP (SAS).

## Results

### General cohort description

General and hormonal characteristics of included patients are provided in Table 1. Age was significantly different among groups, whereas BMI and sex distribution were comparable. Hypertension and dyslipidemia were more frequent in patients with hypercortisolism. The prevalence

**Table 1** General characteristics, clinical and hormonal data of controls and patients with non-secreting adenomas, autonomous cortisol secretion and Cushing's syndrome. Data are expressed as mean with range in parenthesis or frequencies.

	Controls	Non-secreting adenomas	Autonomous cortisol secretion	Cushing's syndrome		P value
				ACTH-independent	ACTH-dependent	
Patients, n	42	27	31	22	27	
General characteristics						
Females, n (%)	31 (73.8)	19 (70.4)	19 (61.3)	16 (72.7)	19 (70.4)	0.830
Age (years)	38.2 (16.0–62.9)	56.8 (31.5–77.0)	63.9 (40.5–87.2)	51.3 (21.0–72.3)	48.2 (16.8–77.8)	<0.001*
BMI (kg/m <sup>2</sup> )	32.7 (17.9–51.3)	31.3 (19.0–45.2)	28.3 (18.4–38.9)	30.4 (20.8–49.7)	28.1 (19.8–46.8)	0.108
Clinical data, n (%)						
Hypertension	23/39 (59.0)	15/27 (55.6)	27/31 (87.1)	17/21 (80.0)	22/27 (81.5)	0.015
Type 2 diabetes	6/41 (14.6)	7/27 (25.9)	9/31 (29.0)	6/21 (28.6)	9/26 (34.6)	0.405
Dyslipidemia	5/31 (16.1)	9/27 (33.3)	11/30 (36.7)	8/21 (38.1)	15/25 (60.0)	0.020
Previous CVD	3/42 (7.1)	4/27 (14.8)	7/31 (22.6)	2/21 (9.5)	4/27 (14.8)	0.406
Hormonal data						
DST (nmol/L)	30.4 (13.8–50.0)	35.9 (19.3–50.0)	129.4 (52.4–744.9)	326.7 (74.5–761.5)	593.2 (102.0–1914.7)	<0.001
ACTH (pmol/L)	2.9 (1.1–6.2)	2.9 (1.3–5.1)	1.8 (0.7–4.8)	1.3 (1.1–3.3)	18.1 (3.8–86.2)	<0.001
LNCS (nmol/L)	0.3 (0.1–0.8)	1.5 (0.1–4.4)	1.3 (0.1–7.7)	4.0 (0.5–16.0)	2.9 (0.6–8.7)	<0.001

Hormonal data are expressed in System International (SI) units. To convert cortisol from the SI units (nmol/L) to the metric units ( $\mu\text{g/dL}$ ), divide by the conversion factor 27.59. To convert ACTH from the SI units (pmol/L) to the metric units (pg/mL), divide by the conversion factor 0.22.

\*All simple contrasts are significant at  $P < 0.05$ , except non-secreting adenomas vs ACTH-independent Cushing's syndrome ( $P = 0.15$ ) and ACTH-independent vs ACTH-dependent Cushing's syndrome ( $P = 0.41$ ).

BMI, body mass index; DST, 1-mg dexamethasone suppression test; LNCS, late-night salivary cortisol.

of diabetes was not significantly different among groups. Hormonal values were different by definition.

### Metabolomic profiles in patients with hypercortisolism

The results of the metabolomics profiling are detailed in [Supplementary Table 1](#) (see section on [supplementary data](#) given at the end of this article). Patients with CS proofed to have lower levels of several amino acids and increased levels of spermidine and taurine, when compared to controls. PC aa C38:3, PC aa C40:4 and PC ae C36:4 were significantly lower than those in controls. Several alterations observed in CS were also present in patients with ACS, i.e. lower levels of histidine, tryptophan, valine and PC aa C38:3 and PC aa C40:4, and increased levels of taurine, in comparison to controls. The comparison of the two subtypes of CS did not highlight relevant differences, apart from increased levels of spermine and taurine, and lower levels of PC ae C36:4, in ACTH-independent CS.

Considering the significant difference in age among the groups, comparison of the metabolite panel was performed after adjustment for age. Following this adjustment ([Supplementary Table 2](#)), patients with CS had significantly lower plasma levels of carnitine, acetyl-carnitine and several acylcarnitines up to C14, when compared to controls. Amino acids, including branched-chain (BCAA) and aromatic amino acids (AAA), and PC aa C38:3, PC aa C38:4 and PC aa C40:4 were significantly lower in plasma of patients with CS, whereas spermidine showed higher values, with respect to controls. The alterations in plasma levels of carnitine, acylcarnitines and phosphatidylcholines were also confirmed in patients with ACS. No difference in plasma levels of sphingomyelins or hexoses was found among groups. The subgroup analysis of the metabolomics profiles between patients with ACTH-independent CS and ACS revealed that only spermidine was significantly higher in the former group ( $P=0.014$ ).

### Relationship between metabolomic profiles and clinical, hormonal and metabolic characteristics

To test whether the presence of diabetes and dyslipidemia could represent a potential confounder in the interpretation of the results, differences among groups were further analyzed in patients without those

co-morbidities. Difference in post-DST and midnight cortisol were firstly analyzed between patients with and without co-morbidities separately in each group, to check whether the hormonal profile could be more severe in the former, generating potential bias in the results. However, no such differences in hormonal values were detectable between patients with diabetes (+/- dyslipidemia) and those without (data not shown). General characteristics and metabolomic profiling of non-diabetic patients ( $n=109$ ) are reported in [Supplementary Table 3](#). After adjustment for age, acetyl-carnitine and hydroxytetradecadienylcarnitine were lower in patients with hypercortisolism (ACS and CS) than in controls, alongside with BCAA, histidine, tyrosine, kynurenine and PC aa C38:3, PC aa C38:4 and PC aa C40:4. Spermidine levels remained higher in plasma of patients with CS than those in controls. Notably, free carnitine, dodecenoylcarnitine and several C14 acylcarnitines were significantly lower in patients with hypercortisolism, when compared to controls, even though at FDR  $\alpha>0.1$ . The alterations of ten of those metabolites were also observed in subjects with neither diabetes nor dyslipidemia ( $n=70$ ), i.e. acetyl-carnitine ( $P=0.004$ ), hydroxytetradecadien-carnitine ( $P=0.001$ ), isoleucine ( $P=0.016$ ), leucine ( $P=0.014$ ), valine ( $P=0.010$ ), tyrosine ( $P=0.007$ ), kynurenine ( $P=0.002$ ), spermidine ( $P<0.001$ ), PC aa C38:3 ( $P=0.009$ ) and PC aa C40:4 ( $P=0.012$ ) (data not shown).

Taking into account the association between metabolites and the different categories of cortisol hypersecretion, a regression model was built to investigate the relationship with post-DST cortisol, including potential contributing/confounding factors ([Table 2](#)). Post-DST cortisol levels were significantly associated with 31 metabolites, irrespective of age, BMI and presence of diabetes and/or dyslipidemia. Notably, several long-chain acylcarnitines  $>C16$  and polyamines were positively associated, whereas amino acids and most phosphatidylcholines  $\geq C38$  were negatively associated with post-DST cortisol. A multinomial logistic regression model was assessed to analyze the relationship between those metabolites and the presence of typical catabolic signs/symptoms of hypercortisolism (myopathy, thin skin, and easy bruising). Overall, histidine was negatively associated with the presence of catabolic signs of CS (odds ratio (OR) 0.02, 95% CI 0.002–0.27;  $P=0.003$ ), whereas spermidine was positively associated (OR 1.93, 95% CI 1.19–3.13;  $P=0.007$ ).



**Table 2** Regression coefficients and *P* values of metabolites (A) and calculated enzymatic activity (B) significantly associated with cortisol after DST. Data are expressed as regression coefficients ( $\beta$ ) with 95% confidence intervals (CI) in parentheses.

	Cortisol after DST (1 $\mu$ g/dL increase)			Age (1 year increase)			BMI (1 kg/m <sup>2</sup> increase)			Type-2 diabetes (presence vs absence)			Dyslipidemia (presence vs absence)		
	$\beta$ (95% CI)	<i>P</i> value		$\beta$ (95% CI)	<i>P</i> value		$\beta$ (95% CI)	<i>P</i> value		$\beta$ (95% CI)	<i>P</i> value		$\beta$ (95% CI)	<i>P</i> value	
<b>A – Metabolites</b>															
Tetradecanoyl-L-carnitine (myristoyl carnitine) (C14)	0.007 (0.002; 0.012)	0.008		0.009 (0.006; 0.013)	<0.001		0.005 (–0.003; 0.014)	0.193		–0.148 (–0.297; 0.001)	0.052		0.075 (–0.055; 0.205)	0.258	
Hexadecanoyl-L-carnitine (palmitoyl carnitine) (C16)	0.009 (0.004; 0.014)	0.001		0.006 (0.002; 0.009)	0.002		0.003 (–0.005; 0.011)	0.403		–0.053 (–0.199; 0.093)	0.479		0.105 (–0.020; 0.229)	0.100	
Hydroxyhexadecanoyl-L-carnitine (C16:1-OH)	0.006 (0.001; 0.011)	0.012		0.008 (0.005; 0.011)	<0.001		0.006 (–0.001; 0.013)	0.071		0.172 (0.048; 0.296)	0.007		0.009 (–0.096; 0.115)	0.863	
Hexadecanoyl-L-carnitine (C16:1)	0.007 (0.003; 0.011)	0.001		0.006 (0.004; 0.009)	<0.001		0.008 (0.002; 0.014)	0.009		–0.104 (–0.216; 0.008)	0.070		0.051 (–0.046; 0.147)	0.304	
Hydroxyhexadecadienyl-L-carnitine (C16:2-OH)	0.004 (0.001; 0.007)	0.011		0.002 (0.001; 0.004)	0.012		0.005 (0.001; 0.010)	0.021		0.025 (–0.054; 0.104)	0.538		0.028 (–0.039; 0.096)	0.409	
Octadecanoyl-L-carnitine (stearoyl carnitine) (C18)	0.008 (0.002; 0.015)	0.009		0.01 (0.006; 0.014)	<0.001		–0.005 (–0.014; 0.004)	0.267		–0.122 (–0.292; 0.048)	0.159		0.134 (–0.009; 0.277)	0.066	
Octadecanoyl-L-carnitine (oleyl carnitine) (C18:1)	0.013 (0.007; 0.020)	<0.001		0.007 (0.002; 0.011)	0.003		0.005 (–0.005; 0.015)	0.368		–0.094 (–0.281; 0.093)	0.324		0.062 (–0.099; 0.222)	0.452	
Octadecadienyl-L-carnitine (linoleyl carnitine) (C18:2)	0.019 (0.010; 0.027)	<0.001		0.003 (–0.002; 0.009)	0.214		–0.001 (–0.012; 0.011)	0.907		–0.173 (–0.394; 0.048)	0.125		0.049 (–0.138; 0.235)	0.609	
Hydroxypropionyl-L-carnitine (C3-OH)	0.003 (0.001; 0.006)	0.005		0.000 (–0.001; 0.002)	0.586		0.004 (0.000; 0.007)	0.032		–0.002 (–0.065; 0.062)	0.958		–0.002 (–0.057; 0.052)	0.929	
Butyryl-L-carnitine (C4)	0.009 (0.002; 0.017)	0.019		0.008 (0.002; 0.013)	0.006		0.010 (–0.004; 0.023)	0.157		0.087 (–0.138; 0.312)	0.447		0.035 (–0.155; 0.225)	0.720	
Hydroxybutyryl-L-carnitine (C4-OH [C3-DC])	0.011 (0.005; 0.017)	0.001		0.009 (0.004; 0.013)	<0.001		0.003 (–0.007; 0.013)	0.578		–0.064 (–0.255; 0.127)	0.512		–0.005 (–0.17; 0.159)	0.952	
Valeryl-L-carnitine (C5)	0.009 (0.003; 0.014)	0.003		0.005 (0.001; 0.009)	0.015		0.012 (0.002; 0.022)	0.016		–0.006 (–0.177; 0.165)	0.946		0.056 (–0.094; 0.205)	0.467	
Fumaryl-L-carnitine (C6 [C4:1-DC])	0.006 (0.001; 0.012)	0.031		0.011 (0.007; 0.015)	<0.001		0.010 (0.001; 0.019)	0.034		–0.022 (–0.183; 0.139)	0.790		0.068 (–0.072; 0.208)	0.340	
Glutaryl-L-carnitine (C5-DC [C6-OH])	0.006 (0.002; 0.010)	0.002		0.007 (0.004; 0.009)	<0.001		0.007 (0.004; 0.008)	0.506		0.079 (–0.029; 0.186)	0.151		–0.036 (–0.129; 0.056)	0.443	
Arginine	–0.010 (–0.016; –0.004)	0.002		0.002 (–0.002; 0.006)	0.393		–0.011 (–0.019; –0.002)	0.017		–0.082 (–0.237; 0.072)	0.297		0.068 (–0.065; 0.201)	0.314	
Histidine	–0.004 (–0.007; 0.000)	0.041		–0.004 (–0.006; –0.002)	0.001		–0.001 (–0.007; 0.004)	0.603		0.050 (–0.047; 0.148)	0.314		–0.025 (–0.110; 0.059)	0.560	
Proline	–0.009 (–0.014; –0.004)	0.001		0.001 (–0.003; 0.004)	0.778		0.004 (–0.003; 0.012)	0.294		0.068 (–0.072; 0.208)	0.341		0.052 (–0.068; 0.171)	0.394	
Tryptophan	–0.006 (–0.010; –0.001)	0.022		–0.005 (–0.009; –0.002)	<0.001		–0.001 (–0.008; 0.006)	0.775		–0.010 (–0.134; 0.113)	0.870		0.028 (–0.078; 0.134)	0.609	
Creatinine	0.006 (0.001; 0.011)	0.028		0.007 (0.003; 0.010)	<0.001		–0.002 (–0.011; 0.006)	0.581		–0.008 (–0.155; 0.14)	0.918		–0.002 (–0.129; 0.126)	0.980	
Methioninesulfoxide	–0.026 (–0.046; –0.007)	0.009		0.012 (–0.002; 0.026)	0.087		0.006 (–0.023; 0.035)	0.675		–0.5800 (–1.064; –0.096)	0.019		0.340 (–0.089; 0.770)	0.120	
Putrescine	0.013 (0.004; 0.022)	0.004		0.002 (–0.004; 0.007)	0.577		–0.003 (–0.015; 0.010)	0.647		–0.145 (–0.37; 0.079)	0.204		0.011 (–0.179; 0.202)	0.908	
Spermidine	0.025 (0.015; 0.036)	<0.001		–0.002 (–0.009; 0.005)	0.640		0.004 (–0.011; 0.019)	0.593		0.098 (–0.176; 0.371)	0.483		–0.084 (–0.318; 0.150)	0.482	
Spermine	0.009 (0.003; 0.015)	0.004		0.000 (–0.004; 0.004)	0.957		0.012 (0.004; 0.021)	0.005		0.160 (0.009; 0.311)	0.038		–0.135 (–0.265; –0.005)	0.042	
PC aa C38:3	–0.010 (–0.016; –0.005)	<0.001		–0.003 (–0.007; 0.001)	0.107		0.004 (–0.004; 0.012)	0.350		0.055 (–0.096; 0.206)	0.473		0.111 (–0.020; 0.241)	0.096	
PC aa C38:4	–0.008 (–0.015; –0.002)	0.007		0.001 (–0.004; 0.005)	0.769		0.003 (–0.007; 0.012)	0.577		0.066 (–0.099; 0.230)	0.435		0.139 (–0.003; 0.281)	0.056	
PC aa C40:2	–0.013 (–0.026; 0.000)	0.047		0.006 (–0.003; 0.016)	0.170		–0.005 (–0.024; 0.013)	0.566		–0.400 (–0.736; –0.064)	0.020		0.142 (–0.145; 0.428)	0.333	
PC aa C40:3	–0.011 (–0.021; –0.001)	0.029		0.004 (–0.003; 0.011)	0.272		–0.011 (–0.025; 0.004)	0.141		–0.222 (–0.481; 0.038)	0.094		0.111 (–0.109; 0.331)	0.323	
PC aa C40:4	–0.010 (–0.016; –0.004)	0.001		–0.003 (–0.007; 0.001)	0.132		–0.002 (–0.011; 0.007)	0.663		0.108 (–0.050; 0.266)	0.179		0.083 (–0.052; 0.218)	0.229	
PC aa C42:4	–0.010 (–0.019; –0.002)	0.019		0.002 (–0.003; 0.008)	0.438		–0.009 (–0.021; 0.003)	0.147		–0.143 (–0.365; 0.078)	0.705		0.111 (–0.079; 0.301)	0.253	
PC aa C42:5	–0.012 (–0.019; –0.005)	0.001		0.000 (–0.005; 0.004)	0.920		–0.014 (–0.024; –0.004)	0.007		–0.030 (–0.214; 0.154)	0.751		0.11 (–0.047; 0.266)	0.170	
PC aa C42:6	–0.008 (–0.015; –0.002)	0.016		–0.001 (–0.005; 0.004)	0.795		–0.014 (–0.023; –0.004)	0.006		–0.010 (–0.189; 0.168)	0.910		0.035 (–0.116; 0.186)	0.649	
<b>B – Calculated enzymatic activity</b>															
Carnitine-palmitoyl-transferase 1 (CPT1)	0.010 (0.005; 0.016)	<0.001		0.002 (–0.002; 0.005)	0.347		–0.003 (–0.011; 0.004)	0.416		–0.05 (–0.189; 0.089)	0.481		0.086 (–0.032; 0.204)	0.153	
Arginase	0.018 (0.009; 0.027)	<0.001		–0.001 (–0.007; 0.006)	0.848		0.013 (–0.001; 0.026)	0.066		0.106 (–0.137; 0.350)	0.391		–0.114 (–0.321; 0.093)	0.281	
Ornithine decarboxylase (ODC)	0.014 (0.003; 0.024)	0.011		–0.004 (–0.010; 0.002)	0.175		–0.006 (–0.020; 0.009)	0.447		–0.112 (–0.371; 0.147)	0.396		–0.054 (–0.28; 0.171)	0.637	
Spermidine synthase	0.010 (0.000; 0.021)	0.054		–0.005 (–0.012; 0.002)	0.159		0.006 (–0.010; 0.022)	0.460		0.091 (–0.177; –0.359)	0.504		–0.056 (–0.279; 0.167)	0.621	
Spermine synthase	–0.019 (–0.027; –0.012)	<0.001		0.000 (–0.006; 0.005)	0.870		0.003 (–0.009; 0.015)	0.672		0.036 (–0.173; 0.244)	0.738		0.013 (–0.168; 0.193)	0.890	

Indirect markers of enzymatic activity were calculated as follows: CPT1, (C16 + C18 acyl carnitines)/carnitine; arginase, ornithine/ornithine; spermidine synthase, spermidine/putrescine; spermine synthase, spermine/spermidine.

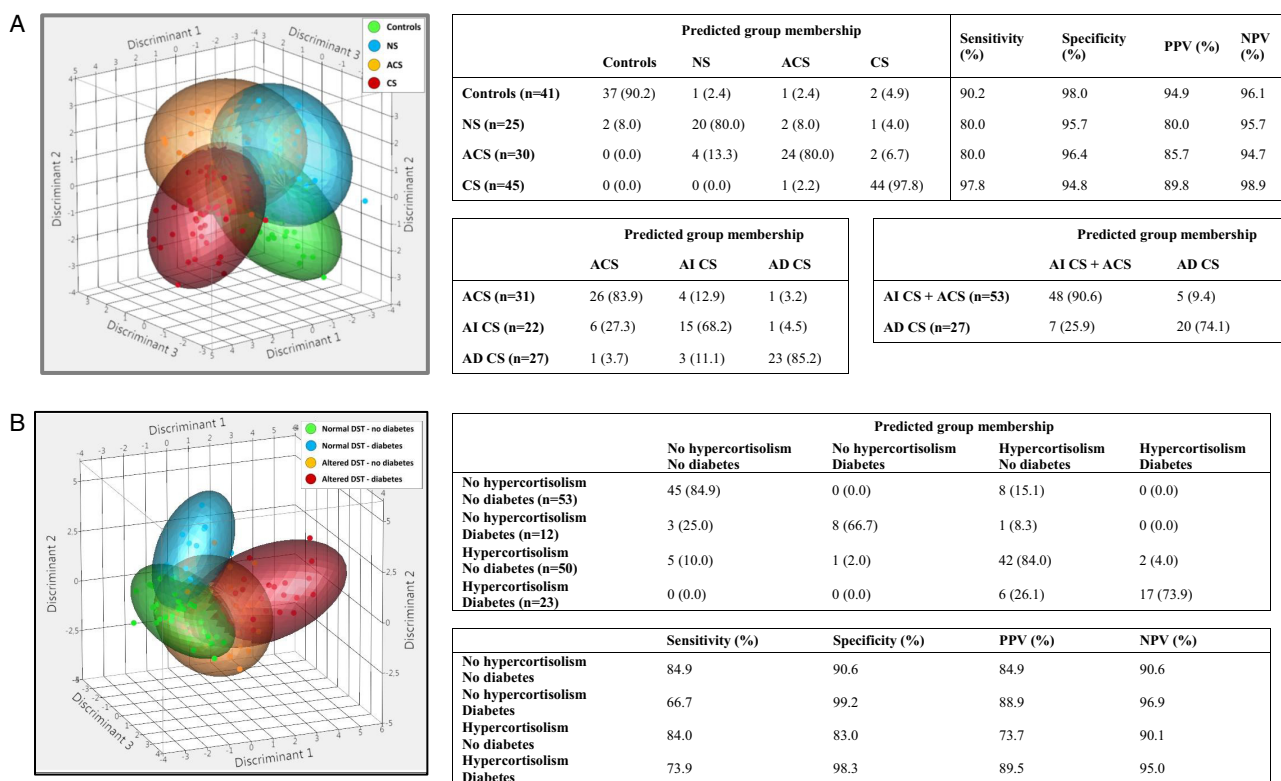
BMI, body mass index; DST, 1-mg dexamethasone suppression test; PC aa, diacyl phosphatidylcholine; SM, sphingomyelin.

Considering the alterations in acylcarnitines and polyamines, we investigated the relationship between post-DST cortisol and metabolite ratios indicative of activity of carnitine-palmitoyl-transferase 1 (CPT-1) and enzymes of the polyamine biosynthetic pathway (arginase, ornithine decarboxylase (ODC), spermidine and spermine synthase). CPT-1, arginase and ODC activities were positively associated with post-DST cortisol, whereas a negative association was found for spermine synthase, independently of confounding factors (Table 2). No significant association was recorded for spermidine synthase ( $P=0.054$ ).

The analyses performed after exclusion of the three patients with ectopic CS did not change the results (data not shown).

### Discriminant analysis for diagnosis and differential diagnosis of CS

Based on a discriminant analysis taking into account the metabolomic panel as well as age, 88.7% of the patients could be correctly classified into the four main diagnostic groups, i.e. CS, ACS, NS and controls (Fig. 1A). The most discriminant metabolites for identification of



**Figure 1**

3D scatter plots of the scores obtained by the discriminant analysis. (A) Discriminant scores for classification of controls, NS, ACS and CS, showing that overall 88.7% of patients were correctly classified. The discriminant analysis also showed a good overall correct classification rate into ACS, AI CS and AD CS (80.0%) and when patients with adrenal hypercortisolism (AI CS+ACS) and AD CS were considered (85.0%). (B) The figure shows the scores obtained by the discriminant analysis for grouping of four novel identified groups, based on the levels of cortisol after DST (normal vs altered) and presence/absence of diabetes. The three scores were able to correctly classify 81.2% of the patients overall. No overlap was observed between diabetic patients with and without alterations of post-DST cortisol, indicating that the metabolic fingerprint of diabetes may be different according to the concomitant presence of hypercortisolism. Data in tables are expressed as numbers with percentages in parentheses. ACS, autonomous cortisol secretion; AD CS, ACTH-dependent CS; AI CS, ACTH-independent CS; CS, Cushing's syndrome; DST, 1-mg dexamethasone suppression test; NPV, negative predictive value; NS, non-secreting adrenocortical adenomas; PPV, positive predictive value.



patients with CS were spermidine, kynurenine, isoleucine and PC aa C40:4. The predictive score calculated by the discriminant coefficient for all those metabolites showed a sensitivity of 77.6% and a specificity of 72.0% in identifying CS (area under the curve – AUC 0.808, 95% CI 0.734–0.883,  $P$  value <0.001). A plot of the ROC curve is shown in [Supplementary Fig. 1](#). Considering the differential diagnosis of endogenous hypercortisolism into ACS, ACTH-independent and ACTH-dependent forms, metabolomic profiling provided an overall classification rate of 80.0%, when restricting the analysis to those cases, and 85.0%, when patients with adrenal hypercortisolism (ACS+ACTH-independent) and ACTH-dependent CS were included ([Fig. 1A](#)). Finally, the analysis was performed to investigate the discriminant power of metabolites for subjects with or without hypercortisolism (post-DST cortisol below or above 50nmol/L) and with or without diabetes respectively. According to this discrimination, 81.2% of the patients were correctly classified ([Fig. 1B](#)).

## Discussion

The present study focused on the quantification of a large panel of metabolites in plasma of patients with hypercortisolism, which was performed for the first time by using a highly accurate MS-based assay. The results of our study clearly indicate that the metabolic profile of patients with hypercortisolism is disturbed involving metabolism of amino acids and polyamines, as well as fatty acid  $\beta$ -oxidation (FAO). Most metabolic alterations were also associated with the severity of cortisol excess, as well as with clinical signs and symptoms of steroid-induced catabolism.

Making usage of the full spectrum of analytes, we assessed the discriminating power of the assay as an aid for patient classification based on a single blood sample drawn in the morning. By using metabolites and age, >80% of patients could be classified between those with CS, ACS, NS and those in whom hypercortisolism had been excluded. Similarly, the profiles could be utilized to separate between ACTH-dependent and independent hypercortisolism in up to 85% of cases. Finally, the metabolomic profile was able to discriminate metabolites mainly associated with cortisol hypersecretion from those related to type-2 diabetes, even though with some overlap between groups. In contrast, no overlap was observed within the group of diabetic patients with and without alterations of post-DST cortisol, indicating that the metabolic fingerprint of diabetes differs according to the concomitant presence of hypercortisolism.

Age-adjusted analysis revealed alterations in amino acids and polyamines in patients with hypercortisolism, which were well correlated with post-DST cortisol levels. The reduction of BCAA (isoleucine, leucine and valine) was among the most frequently observed characteristics. BCAA are known to be increased proportionally to the muscle mass in non-fed states, to provide substrates for energy production, and to be reduced in severe muscle atrophy ([13](#)). Their lower levels in patients with hypercortisolism may uncover a significant reduction of muscular mass, a well-known consequence of hypercortisolism and one of the most specific clinical characteristics of patients with endogenous and iatrogenic CS ([3](#)). BCAA reduction in hypercortisolism was even more marked after removing the confounding effect of diabetes, which is associated with disruption of amino acid metabolism and proximal muscle atrophy ([14](#)). Interestingly, studies on animal models highlighted a role for BCAA in reversing dexamethasone-induced muscle atrophy, shedding light on the interplay between glucocorticoid and BCAA in maintaining CS-related sarcopenia ([15](#)). Several AAA were also decreased during hypercortisolism. Histidine plays major roles as a component of collagen and as a precursor of histidine-containing dipeptide beta-alanyl-L-histidine (carnosine) in human skeletal muscle ([16](#)). In our cohort, histidine was inversely associated with the severity of hypercortisolism and showed a negative correlation with clinically relevant signs and symptoms such as proximal muscle weakness, skin thinning and easy bruising. These findings support the hypothesis that histidine might represent a marker and a potential mediator of cortisol-related alterations of collagen and skeletal muscle. Disruption of tryptophan metabolism may also contribute to steroid-dependent skeletal muscle alteration. Given that tryptophan and its metabolite kynurenine have shown positive effects on the expression of myogenic markers in animal models ([17](#)), reduced levels of those compounds may represent a pejorative factor contributing to cortisol-induced reduction of protein synthesis. Due to its ability to cross the blood–brain barrier ([18](#)), lower plasma tryptophan levels may also be associated with a reduced intracerebral serotonin production. Whether this alteration could contribute to CS-related depression is an intriguing hypothesis that deserves further studies.

Polyamines are low-molecular-weight, positively charged compounds acting as mediators of several biological processes, mainly cell growth, response to oxidative stress ([19](#)) and cortisol-induced inflammatory response ([20](#)). Polyamines are produced by *de novo* synthesis from proline, glutamine and arginine, with

ornithine decarboxylase (ODC) as the rate-limiting step. Spermidine levels were significantly increased in patients with CS and polyamines correlated with the severity of hypercortisolism. Furthermore, spermidine was clearly associated with catabolic signs and symptoms of hypercortisolism. Increased polyamine production could rely on the direct activation of ODC by cortisol, given that the ODC promoter contains hormonally regulated transcription factor response elements (21) and based on animal studies demonstrating that ODC is activated by dexamethasone (22). In our cohort, indirect measures of ODC and arginase activity were positively associated with post-DST cortisol levels, indicating arginine as a potential source for ornithine generation in hypercortisolism and a cortisol-related ODC activation. Conversely, the inverse association with spermidine/spermine ratio may represent either an overload of the non-inducible spermine synthase or an increased catabolism of spermine through N-acetyl-spermine. The role of polyamines in human diseases has recently been claimed in several studies, showing that their production was increased in models of experimentally induced cardiac hypertrophy (23) and that spermidine had detrimental effects on cardiomyocytes under hypoxic stress (24). Accordingly, polyamines were also found increased in patients with heart failure (25). Considering these results and previous studies demonstrating interplay between cortisol and polyamines during inflammatory processes (20), the novel finding on perturbation of polyamine metabolism in hypercortisolism is interesting. The effects of their elevation on the cardiovascular system and on inflammation should be further clarified in targeted studies.

Alterations in carnitine and acylcarnitines point toward disturbances of FAO in patients with hypercortisolism, as also shown in previous studies (26). During fed-to-fast transition, the switch from glucose to fatty acid metabolism leads to increased uptake of carnitine and long-chain fatty acids, which are combined by CPT1 to generate acylcarnitines and internalized in the mitochondrial matrix through the carnitine shuttle, initiating FAO and producing acetyl-CoA to sustain the Krebs cycle. When excessive, acetyl-CoA is converted to the membrane-permeable acetyl-carnitine by the carnitine acetyltransferase (CRAT) and exported. The reduced levels of acylcarnitines up to C14 and the relative increase of >C16 acylcarnitines, associated with post-DST cortisol levels, point toward an incomplete FAO in patients with hypercortisolism. The increased availability of free fatty acids due to cortisol-induced lipolysis (3) may represent the trigger for the metabolic overload

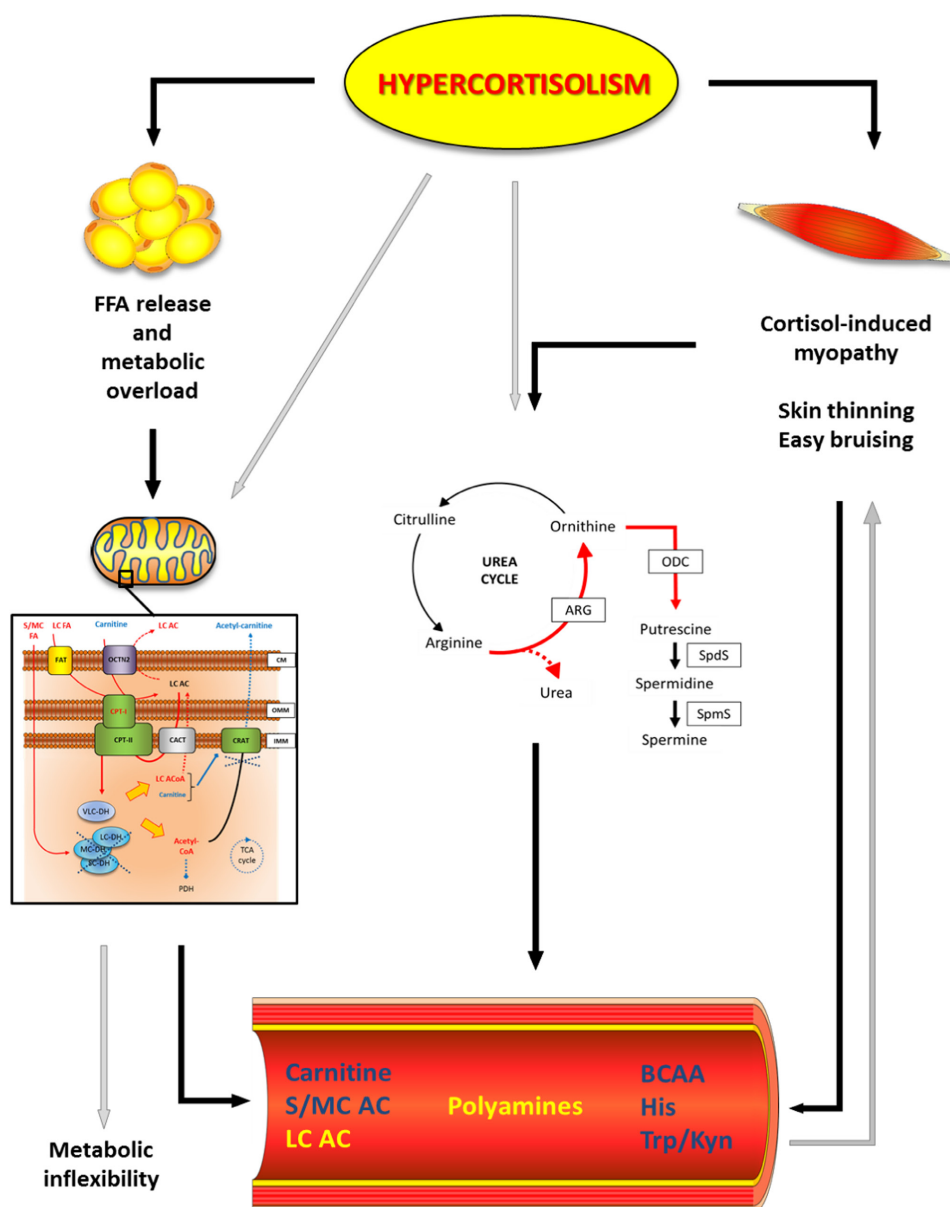
to mitochondria and depletion of components of the Krebs cycle, similar to the cascade of events postulated as a causative mechanism in type-2 diabetes (27). The low levels of plasma acetyl-carnitine in hypercortisolism provide additional clues. Considering that intra-matrix accumulation of long-chain acyl-CoA is an inhibitory signal for CRAT (28), it is conceivable that the sustained cortisol-driven metabolic overload may lower CRAT activity. Therefore, the reduced acetyl-carnitine levels may uncover an increase in intra-matrix FAO-derived acetyl-CoA, which, in turn, inhibits pyruvate dehydrogenase and glycolytic processes (29). Additionally, *in vitro* studies have demonstrated inhibitory effects of glucocorticoids on enzymes of FAO (long-, medium- and short-chain acyl-CoA dehydrogenases), without affecting the activity of very-long-chain acyl-CoA dehydrogenases (30). These results give feed to the hypothesis that the incomplete FAO in patients with hypercortisolism may rely on a selective inhibition at C14 fatty acyl-CoA, due to direct cortisol-dependent effects.

Interestingly, no alteration of SM levels was identified among groups in all comparisons. This result may indicate that composition of plasma membrane as well as activities of enzymes of the SM cycle and ceramide levels may not be affected by hypercortisolism. However, further targeted studies are required to unravel this complex aspect.

We could not analyze the potential effects of androgens on the different metabolomic profiles in patients with ACTH-dependent vs ACTH-independent CS because of the lack of a sufficient number of data to perform a reliable statistical analysis. This intriguing aspect should be investigated in targeted future studies.

A summary of the main metabolic alterations in patients with hypercortisolism is illustrated in Fig. 2. Levels of amino acids and polyamines are consistent with prolonged muscular catabolism and inhibition of protein synthesis in the context of sustained hypercortisolism. Interestingly, several alterations were replicated in patients with ACS, leading to the intriguing hypothesis that some degrees of protein catabolism and muscular atrophy could be relevant also in patients without classical clinical signs of CS. Moreover, the metabolic profiling of acylcarnitines provides indirect evidences that the metabolic inflexibility (the impossibility to switch from fatty acid to glucose oxidation and vice versa) (31), described in patients with type-2 diabetes, may be a characteristic feature of hypercortisolism.

Beside the potential mechanisms underlying the differences among groups, which should be considered speculative, mainly due to the cross-sectional design, this



**Figure 2**

Summary of the most important plasma metabolomic profile alterations identified in association with hypercortisolism and speculative hypothesis on the main pathogenetic pathways involved. Black arrows indicate known pathways altered by hypercortisolism, whereas gray arrows point at potential novel pathways. In the left box (summarizing the altered steps of fatty acid  $\beta$ -oxidation) and in the cartoon of the urea cycle, pathways activated by hypercortisolism are highlighted in red, whereas downregulated pathways are shown in light blue. Dotted lines indicate potential novel pathways. Metabolites listed in the blood vessel are marked in blue and yellow according to their levels in plasma (reduced vs increased respectively). ARG, arginase; BCAA, branched chained amino acids; CACT, carnitine–acylcarnitine translocase; CM, cell membrane; CPT, carnitine-palmitoyl-transferase; CRAT, carnitine acetyl-CoA transferase; FAT, fatty acid transporter; FFA, free fatty acids; His, histidine; IMM, inner mitochondrial membrane; Kyn, kynurenine; LC AC, long-chain acylcarnitines; LC ACoA, long-chain acyl-CoA; LC-DH, long-chain acyl-CoA dehydrogenase; LC FA, long-chain fatty acids; MC-DH, medium-chain acyl-CoA dehydrogenase; ODC, ornithine decarboxylase; OMM, outer mitochondrial membrane; PDH, pyruvate dehydrogenase; SC-DH, short-chain acyl-CoA dehydrogenase; S/MC FA, short/medium-chain fatty acids; SpdS, spermidine synthase; SpmS, spermine synthase; TCA cycle, tricarboxylic acid cycle; Trp, tryptophan; VLC-DH, very long-chain acyl-CoA dehydrogenase.

study provides information about several specific markers that can be identified in patients with hypercortisolism. Therefore, the results of this study should be interpreted in the light of a basis for future research investigating a more targeted panel of metabolites in a larger population, in a prospective setting. Moreover, the results obtained in this unique model of hypercortisolism provide an invaluable basis to unravel the metabolic correlates of patients under chronic glucocorticoid treatment, which are often difficult to be interpreted due to interferences by the underlying glucocorticoid-requiring pathological condition. Additionally, the possibility to use a restricted panel of metabolites for identifying patients with cortisol-related metabolic alterations may have a high impact on the classification of ACS, which still relies on a non-standardized combination of hormonal tests.

Future studies are needed to prospectively assess the relationship between metabolic alterations and co-morbidities progression, especially in ACS, and the utility of nutritional support (e.g. carnitine and targeted amino acids supplementation) in patients with persistent/recurrent CS and those under chronic glucocorticoid treatment.

#### Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-17-0109>.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

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#### Author contribution statement

Collection of clinical and hormonal data, and blood samples were done by G D D, M Q, T D, N R, M K, C M B, G S, M F, M R and F B. Literature research and data analysis were performed by G D D, C P, J A and F B. Metabolomic profiling was done by C P and J A. Statistical analysis was performed by G D D. All authors contributed to data interpretation and writing of the manuscript.

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